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Short communication

Transcriptional expression levels of chicken collectins are affected by avian influenza A virus inoculation

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ABSTRACT

Mammalian collectins have been found to play an important role in the defense against influenza A virus H9N2 inoculation, but for chicken collectins this has not yet been clarified. The aim of this study was to determine the effect of avian influenza A virus (AIV) inoculation on collectin gene expression in the respiratory tract of chickens and whether this was affected by age. For this purpose 1- and 4-week-old chickens were inoculated intratracheally with PBS or H9N2 AIV. Chickens were killed at 0, 8, 16 and 24 h post-inoculation and trachea and lung were harvested for analysis. Viral RNA expression and mRNA expression of chicken collectins 1 and 2 (cCL-1 and cCL-2), chicken lung lectin (cLL) and chicken surfactant protein A (cSP-A) were determined using real-time quantitative RT-PCR. In lung, a decrease in mRNA expression of cCL-2, cLL and cSP-A after inoculation with H9N2 was seen in both 1- and 4-week-old birds, although at different time points, while in trachea changes were only seen in 4-week-old birds and expression was increased. Moreover, collectin expression correlated with viral RNA expression in lung of 1-week-old birds. These results suggest that both age and location in the respiratory tract affect changes in collectin mRNA expression after inoculation with H9N2 and indicate a possible role for collectins in the host response to AIV in the respiratory tract of chickens.

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1. Introduction

The first line of defense against invading pathogens in the respiratory tract is provided by the innate immune system. This phylogenetically ancient system does not require previous exposure to a pathogen and acts within hours after the first encounter. Especially in the early stages of life, the host is highly dependent on innate immunity for protection because the adaptive immune system is not fully developed yet. An important group of effector molecules of the innate immune system comprises collectins, proteins characterized by a collagen- and a C-type lectin domain. These proteins are pattern recognition

molecules that can bind and neutralize a wide array of pathogens including bacteria and viruses (reviewed by Hogenkamp et al., 2007).

For mammalian collectins, several *in vitro* studies have described a strong neutralizing activity against influenza A virus (Hartshorn et al., 1997; Van Eijk et al., 2003; White et al., 2008). Collectins are thought to bind carbohydrate residues present on influenza hemagglutinin and neuraminidase, thereby aggregating viral particles and preventing invasion of host cells (Reading et al., 2007). In addition, a highly reduced clearance of influenza virus was observed in mice lacking either surfactant protein A or surfactant protein D, implicating the importance of these mammalian collectins in defense against influenza virus inoculations *in vivo* (Sano and Kuroki, 2005). In chicken, five collectins have been described thus far, which are mannan binding lectin (Laursen et al., 1998), surfactant

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protein A (cSP-A) and chicken collectins 1, 2 and 3 (cCL-1–3) (Hogenkamp et al., 2006). In addition, a chicken lung lectin (cLL) with high sequence homology to cSP-A was reported. Whether chicken collectins can protect against influenza A virus inoculation has not been clarified, but cLL has been shown to possess *in vitro* hemagglutination inhibiting activity (Hogenkamp et al., 2008).

Avian influenza virus (AIV) enters the respiratory epithelium using sialic acid receptors linked to galactose by an α -2,3 linkage, although the pattern of receptor distribution in chickens is less defined than in mammals (Wan and Perez, 2006). H9N2 AIV has been shown to have a preference for infecting the upper part of the respiratory tract (Nili and Asasi, 2002). After spray inoculation, viral replication was detected at 24 h post-inoculation in both trachea and lung of chickens. Virus was first detected in the epithelium of the trachea, intrapulmonary bronchus and in adjacent parabronchi, and thereafter spread to other parabronchi deeper in the lung (Reemers et al., 2009).

The aim of the current work was to determine changes in gene expression of chicken collectins upon AIV inoculation in the respiratory tract of 1- and 4-week-old chickens and the effect of age on collectin gene expression after AIV inoculation.

2. Materials and methods

2.1. Inoculation model

AIV, subtype H9N2, isolate A/Chicken/United Arab Emirates/99 was kindly provided by Intervet Schering-Plough Animal Health, Boxmeer, The Netherlands. The experiment was carried out according to protocols approved by the Animal Experiment Committee of Utrecht University (The Netherlands). One- and four-week-old Lohmann Brown layer chickens were inoculated intratracheally with either 0.1 ml PBS or $10^{7.7}$ EID₅₀ H9N2. Before inoculation 5 control chickens per age were killed and at 8, 16 and 24 h post-inoculation (p.i.) 5 control and 5 H9N2 inoculated chickens per age were killed. The cranial part of the lung containing the intrapulmonary bronchus and the upper trachea were used for analysis.

2.2. Real-time quantitative RT-PCR (qRT-PCR)

Lung and trachea were homogenized and total RNA was isolated using the RNeasy Mini Kit and DNase treated (Qiagen Benelux B.V., Venlo, The Netherlands) as described previously (Reemers et al., 2009). cDNA was generated with reverse transcription using the iScript cDNA Synthesis Kit (Biorad Laboratories B.V., The Netherlands).

Detection of GAPDH, H9 hemagglutinin (HA) and 28S products was performed as described by Reemers et al. (2009). Real-time qRT-PCR was performed for cCL-1, cCL-2, cLL and cSP-A using primers and probes depicted in Table 1 according to the following cycle protocol: 2 min at 50 °C, 10 min at 95 °C (denaturation); 40 cycles: 15 s at 95 °C and 60 s at 60 °C. GAPDH was used as a reference gene for correction of viral HA RNA expression and 28S as reference gene for collectin mRNA expression. Corrections for variation in RNA preparation and sampling were performed as previously described (Reemers et al., 2009). Results are expressed in terms of the threshold cycle value (Ct) and given as corrected 40-Ct values.

2.3. Statistical analysis

Significance between H9N2 inoculated and control samples and between age groups within a time point was determined with an ANOVA. Significance between time points within age groups and treatment groups was determined with an ANOVA and Tukey post hoc test. Correlation between viral RNA and collectin mRNA expression was based on the Pearson correlation coefficient (*r*) and determined using SPSS 15.0 software. Data were expressed as means with standard error of the mean (SEM). A *p*-value <0.05 was considered significant.

3. Results

3.1. Viral RNA expression in lung and trachea

Viral RNA expression was detected in lung and trachea of all H9N2 inoculated birds (Fig. 1). There was no significant difference in viral RNA levels in lung between

Table 1
Real-time qRT-PCR primers and probes.

Target	Probe or primer ^a	Sequence (5'–3')	Accession no. ^b
cCL-1	F	5'-ATTGTCAAAGAAGAGAAGAATTACAGAG-3'	DQ129668
	R	5'-GAGGAGATGTAATCAGCAAGCAG-3'	
	Probe	5'-(FAM)-CGTTGGTCACCTCATCTTTAGGCATGGC-(BHQ)-3'	
cCL-2	F	5'-GGGAGCCCAACAATGCCTATG-3'	DQ129669
	R	5'-GTAATATGACATGCAACATCATTCCAC-3'	
	Probe	5'-(FAM)-TGCCACCATCTCCACACAGTCCTCCTC-(BHQ)-3'	
cLL	F	5'-CTTACAAGGGAAGATAACAAAGTCTGG-3'	DQ129667
	R	5'-CATTCTTGGAGATGCAATACACC-3'	
	Probe	5'-(FAM)-CTCTGGCACAATTTACCGTAGCATGG-(BHQ)-3'	
cSP-A	F	5'-GGAATGACAGAAGGTGCAATCAG-3'	AF411083
	R	5'-GCAATGTTGAGTTTATTAGCTACAAATG-3'	
	Probe	5'-(FAM)-CCGCCTTGTGTCTGCCAGTTTATGTGC-(BHQ)-3'	

^a F, forward; R, reverse.

^b Genomic DNA sequence.

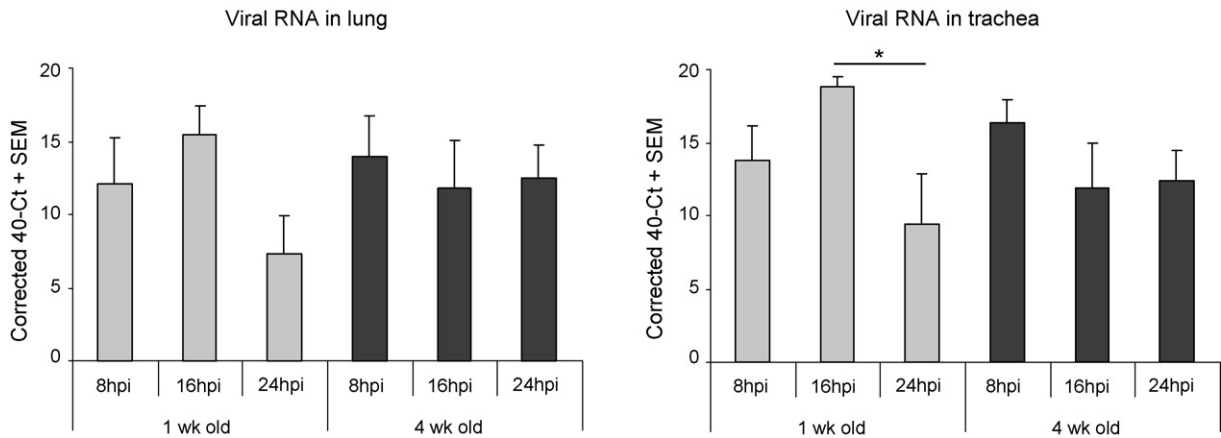


Fig. 1. Viral RNA expression in 1- and 4-week-old H9N2 inoculated birds at 8, 16 and 24 h p.i. in lung and trachea determined with real-time qRT-PCR. *Significant difference in viral RNA expression between 16 and 24 h p.i. in 1-week-old birds ($p < 0.05$).

1- and 4-week-old birds within a time point or between time points within an age group. In trachea there was no significant difference between 1- and 4-week-old birds within a time point. Only the decline of viral RNA expression in trachea of 1-week-old birds at 24 h p.i. was significantly different from the expression at 16 h p.i.

3.2. Collectin mRNA expression in lung and trachea

Expression of cCL-1, cCL-2, cLL and cSP-A mRNA was determined in individual birds at all time points in both lung and trachea (Figs. 2 and 3). cCL-2, cLL and cSP-A mRNA levels in lung of H9N2 inoculated birds were generally

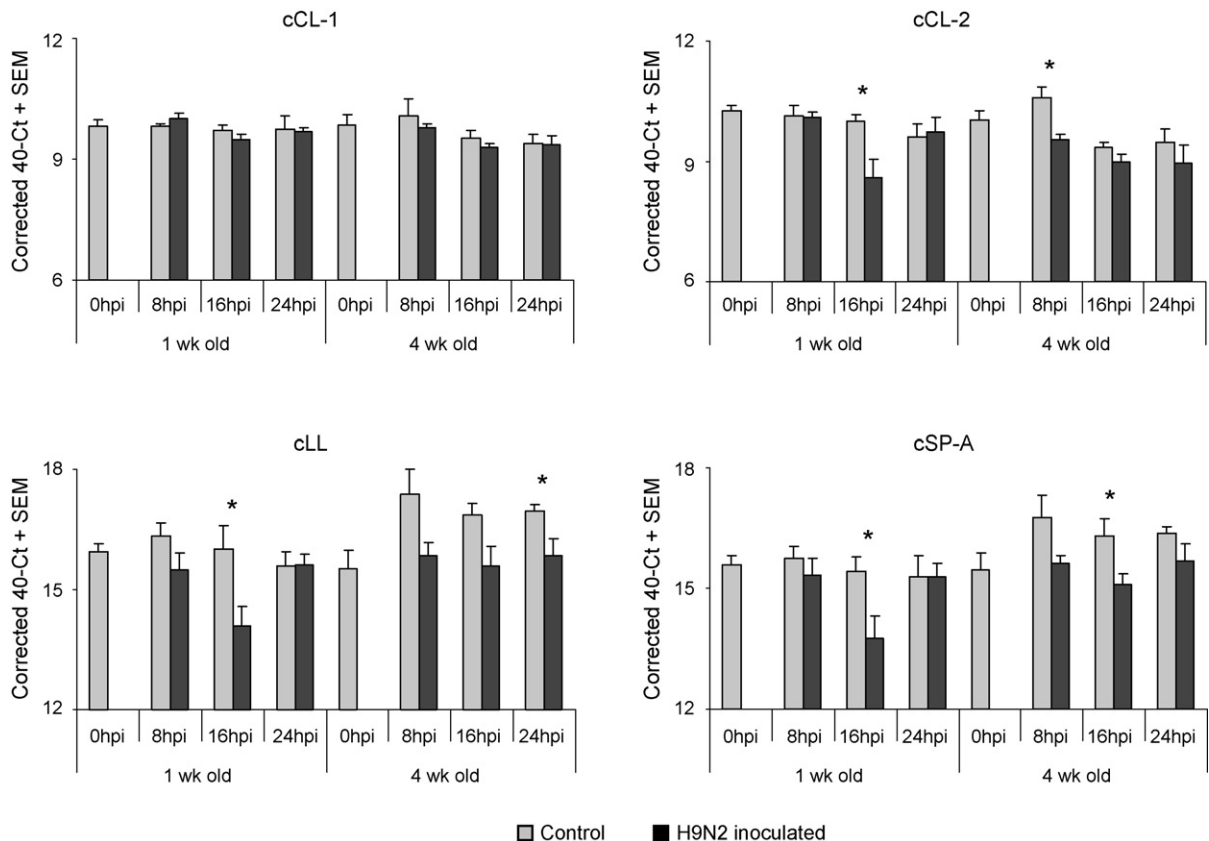


Fig. 2. cCL-1, cCL-2, cLL and cSP-A mRNA expression in lung of 1- and 4-week-old birds at 0, 8, 16 and 24 h p.i. determined with real-time qRT-PCR. *Significant difference in collectin mRNA expression between control and H9N2 inoculated birds ($p < 0.05$).

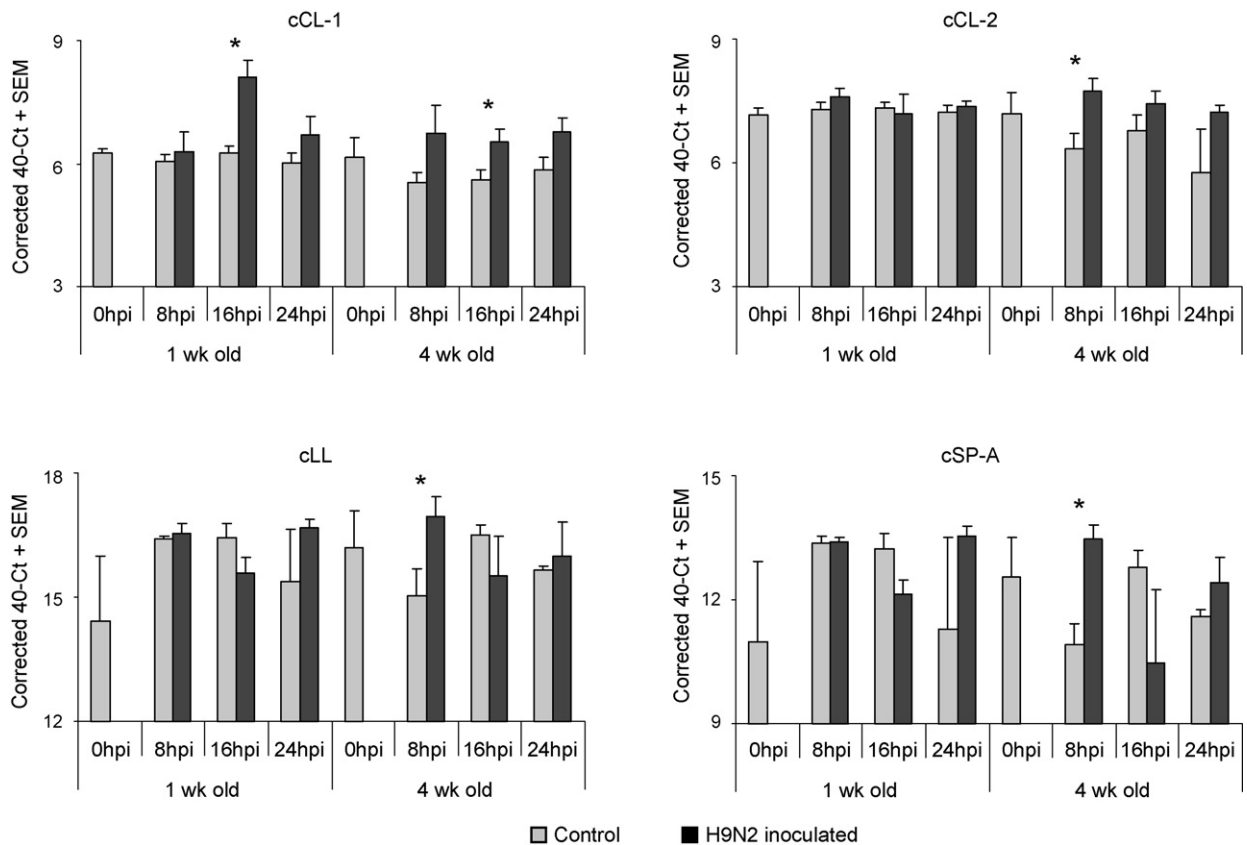


Fig. 3. cCL-1, cCL-2, cLL and cSP-A mRNA expression in trachea of 1- and 4-week-old birds at 0, 8, 16 and 24 h.p.i. determined with real-time qRT-PCR. *Significant difference in collectin mRNA expression between control and H9N2 inoculated birds ($p < 0.05$).

lower compared to control birds. In 1-week-old birds, cCL-2, cLL and cSP-A mRNA levels were significantly decreased after H9N2 inoculation at 16 h p.i. In 4-week-old birds, there was a significant decrease in mRNA levels of cCL-2 at 8 h, cSP-A at 16 h and cLL at 24 h p.i. between lung of H9N2 inoculated and control birds.

Although expression in lung was mostly decreased after H9N2 inoculation, collectin mRNA expression in trachea was mostly increased in H9N2 inoculated birds. The cCL-1 mRNA level was significantly higher at 16 h p.i. in H9N2 inoculated birds compared to control birds in trachea of both 1- and 4-week-old birds. In 4-week-old birds, cCL-2, cLL and cSP-A mRNA levels were significantly increased at 8 h p.i. after H9N2 inoculation. All observed changes were between 1 and 2 Ct values corresponding to a 2–4-fold change in mRNA levels.

3.3. Correlations between viral RNA and collectin mRNA expression in lung and trachea

First the correlation between viral RNA and collectin mRNA expression was determined per age group for all collectins in lung and trachea over time. If a correlation is found this means that viral RNA expression and collectin mRNA expression correlate at every time point. There was no significant correlation between viral RNA expression and mRNA expression of any of the collectins in lung and

trachea of 4-week-old birds over time. No significant correlation was seen in trachea of 1-week-old birds for any of the collectins, however in lung there was a significant strong negative correlation over time between viral RNA expression and mRNA expression of cCL-2 ($r = -0.668$, $p = 0.007$), cLL ($r = -0.655$, $p = 0.008$) and cSP-A ($r = -0.657$, $p = 0.008$).

Although no correlation was found between viral RNA and collectin mRNA expression in trachea of 4-week-old birds over time, there was a significant difference in collectin mRNA expression after H9N2 inoculation at certain time points. Only cCL-2 mRNA expression correlated with viral RNA expression at 8 h p.i.

4. Discussion

The aim of this study was to determine changes in chicken collectin gene expression due to AIV inoculation in the chicken respiratory tract and whether this was affected by age. Collectins have only relatively recently been described in the chicken and their role in the innate immune response is largely unknown. Based on sequence homology and gene expression profiles, two chicken homologues for SP-A were found: cSP-A and cLL. However, the chicken genome did not seem to contain a homologue for the second pulmonary collectin SP-D. In addition, chicken collectins 1, 2 and 3 were found which resemble

the mammalian collectin liver 1 (CL-L1) (Ohtani et al., 1999), collectin kidney 1 (CL-K1) (Keshi et al., 2006) and collectin placenta 1 (CL-P1) (Ohtani et al., 2001). The biological roles of CL-L1 and CL-K1 have not been determined yet, leaving no indication for a possible role of chicken collectins cCL-1 and cCL-2. However, both cCL-1 and cCL-2 are expressed in the chicken respiratory tract (Hogenkamp et al., 2007). The cCL-3 homologue CL-P1 is different from other collectins in the way that it is a type II membrane protein. It acts as a scavenger receptor on endothelial cells, while all other collectins are soluble effector molecules involved in direct neutralization of pathogens. A role for cCL-3 in direct neutralization of viruses in the lung is therefore unlikely.

Based on this similarity to mammalian collectins and the location of expression in the chicken we investigated the changes in mRNA expression of cSP-A, cLL, cCL-1 and cCL-2 after an H9N2 AIV inoculation in the chicken respiratory tract. In the lung of 1-week-old birds, a down regulation was observed for cLL, cSP-A and cCL-2 mRNA, at 16 h p.i. This time point significantly correlated to the observed peak in viral load in the lung. In addition the expression of cCL-2 and cSP-A mRNA was down regulated in 4-week-old birds at 8 and 16 h p.i., but no correlation to viral RNA expression was found. This indicates that expression of cSP-A, cLL and cCL-2 mRNA expression is affected by H9N2 inoculation early after inoculation and in 1-week-old birds is directly related to viral RNA expression in lung. Interestingly, in the trachea, all significant changes due to H9N2 inoculation were related to up regulation of collectin mRNA expression, suggesting host collectin responses after H9N2 inoculation are site specific. cCL-1 was up regulated at 16 h p.i. in trachea of both 1- and 4-week-old birds, while cCL-1 mRNA expression was not affected by H9N2 inoculation in lung. This suggests that changes of cCL-1 mRNA expression after H9N2 inoculation are dependent on the location in the respiratory tract. cCL-2 expression was affected by H9N2 inoculation in 4-week-old birds at 8 h p.i. in both lung and trachea, although down regulated in lung and up regulated in trachea, indicating that cCL-2 mRNA expression changes time specifically at 8 h p.i. after H9N2 inoculation. In lung, changes in mRNA expression of cCL-2, cLL and cSP-A after H9N2 inoculation were seen in both 1- and 4-week-old birds, although at different time points, while in trachea changes were only seen in 4-week-old birds. This suggests that both age and location in the respiratory tract affect changes in collectin mRNA expression after H9N2 inoculation, though one has to keep in mind that changes found at early time points are caused by the virus inoculum whereas at 24 h p.i. viral replication might affect the outcome. In mammals site specific expression and secretion of collectins in the airways has been reported (Coalson et al., 1998; Rooney, 2001). Furthermore, down regulation of SP-A gene expression through p38 MAPK and PI-3 kinase pathways have been described in lung epithelial cells (Miakotina et al., 2002a; Miakotina and Snyder, 2002b). Epithelial cells in the lung differ from tracheal ciliated epithelial cells supporting the possibility of site specific collectin expression after H9N2 inoculation in the chicken. Whether similar signalling pathways are involved in regulation of

chicken collectins needs to be addressed to clarify our observed tissue specific changes of these collectins.

Without knowing the exact function of chicken (col)lectins in innate defense, it is difficult to relate the observed changes in gene expression to a biological effect. In mammals, an up regulation of collectins has frequently been observed in both bacterial and viral infections (Murray et al., 2002; Grubor et al., 2004), which is usually interpreted as a direct response of the host to increase its defense against the incoming pathogens. A down regulation of collectins upon infection is less frequently reported, but could reflect a survival strategy of the pathogen to overcome the host's hostile environment, as has been described for other innate immune proteins such as Toll-like receptors and defensins (Wang et al., 2000; Shin et al., 2007). However, whether this applies to AIV in the chicken lung and why the altered expression of chicken (col)lectins is tissue specific is not clear at this moment and requires further investigation.

In conclusion, the results obtained in this study show that mRNA expression of chicken collectins cCL-1, cCL-2, and cSP-A and cLL are affected by H9N2 AIV inoculation. The effect is tissue specific, showing up regulated mRNA expression in the trachea and down regulation in the lung. Furthermore, changes in collectin mRNA expression are age specific, showing differential gene expression in 1- and 4-week-old birds. These observed changes in collectin mRNA expression implicate that chicken collectins can play an important role in innate defense against viral infection, especially in neonates. More research is needed to relate the changes in collectin mRNA expression after H9N2 AIV inoculation to a biological function and clarify the function of collectins within the innate response to infection.

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